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The Small GTP-Binding Protein rab4 Controls an Early Sorting Event on the Endocytic Pathway

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Summary

rab4 is a ras-like GTP-binding protein that associates with early endosomes in a cell cycle–dependent fashion. To determine its role during endocytosis, we generated stable cell lines that overexpressed mutant or wild-type rab4. By measuring endocytosis, transport to lysosomes, and recycling, we found that overexpression of wild-type rab4 had differential effects on the endocytic pathway. Although initial rates of internalization and degradation were not inhibited, the transfectants exhibited a 3-fold decrease in fluid phase endocytosis as well as an alteration in transferrin receptor (Tfn-R) recycling. Wild-type rab4 caused a redistribution of Tfn-R's from endosomes to the plasma membrane. It also blocked iron discharge by preventing the delivery of Tfn to acidic early endosomes, instead causing Tfn accumulation in a population of nonacidic vesicles and tubules. rab4 thus appears to control the function or formation of endosomes involved in recycling.

Introduction

ras-related GTP-binding proteins comprise a family of low molecular size (20–30 kd) polypeptides that are thought to serve critical regulatory roles in an increasing number of intracellular events. The identification in yeast of mutant forms of these proteins has provided direct evidence for their importance in membrane traffic. These mutants, sec4 and ypt1, are blocked in two distinct steps of the secretory pathway corresponding to fusion of secretory vesicles with the plasma membrane and transport from the endoplasmic reticulum to the Golgi, respectively (Bacon et al., 1989; Goud et al., 1988). Precise mechanisms are unknown, but it is thought that these proteins ensure the specificity or directionality of a vesicle budding or fusion event by coupling the completion of the reaction to the hydrolysis of bound GTP (Bourne, 1988).

Although the activities of GTP-binding proteins in animal cells are even less well defined, numerous members of this family have now been identified and classified into several groups on the basis of sequence similarities (Valencia et al., 1991). Perhaps the largest single group is represented by the rab proteins, which consists of >20 members (Chavrier et al., 1990b; Zahraoui et al., 1989). Importantly, many of these proteins have been localized to a variety of distinct intracellular compartments, suggesting that different rab proteins may be responsible for individual steps of intracellular transport (Balch, 1990; Goud and McCaffrey, 1991; Pfeffer, 1992). In the endocytic pathway, rab4 and rab5 have been localized to early endosomes, while rab7 has been found with late endosomes; GTP-binding proteins have yet to be identified on lysosomes (Chavrier et al., 1990a; van der Sluijs et al., 1991). Although the functions of the endosome-associated rabs are not clear, antibodies to rab5 block homotypic fusion between early endosomes in vitro (Gorvel et al., 1991). This inhibition was reversed by adding cytosol from cells transiently overexpressing wild-type rab5 but not a rab5 mutant deficient in the ability to bind GTP. Despite the fact that the functional significance of endosome fusion during endocytosis is unknown, this result demonstrates that ras-related GTP-binding proteins are likely to be important in the endocytic as well as the secretory pathway.

The fact that early endosomes are associated with at least two rab proteins is consistent with the fact that these organelles engage in multiple transport and sorting events, each possibly requiring distinct GTP-binding proteins. Incoming coated vesicles deliver internalized ligands and solutes to acidic early endosomes, which then mediate the sorting and recycling of some receptors and ligands back to the plasma membrane or the transport of other receptors and ligands to late endosomes and lysosomes for degradation (Gruenberg and Howell, 1989; Helenius et al., 1983; Hopkins, 1992; Kornfeld and Mellman, 1989). Recycling appears to involve the accumulation of receptors in the tubular extensions of early endosomes, while transport to lysosomes correlates with ligand accumulation in the more vesicular portions of the endosome (Geuze et al., 1983, 1987; Hopkins et al., 1990; Marsh et al., 1986). Given even these minimal functions, there are multiple fusion, budding, or other events that may involve distinct GTP-binding proteins. Such heterogeneity would be a basic, but as yet untested, prediction of whether individual rab proteins exhibit specificity of function in addition to specificity of intracellular localization.

Consequently, it is of importance to determine the role of rab4 on the endocytic pathway and to establish whether its function differs in any way from that of rab5. Since it is not clear which, if any, cell-free assay for endocytosis might be rab4 dependent and since the function of any rab protein has yet to be demonstrated in intact cells, we chose to search for specific alterations in cells stably expressing wild-type or mutant rab4. Our results demonstrate that rab4 plays an important role in an early sorting event during endocytosis. Overexpression of wild-type rab4 restricted access of incoming transferrin receptor (Tfn-R) to typical early endosomes, causing transferrin (Tfn) accumulation in a population of nonacidic vesicles and tubules possibly involved in recycling. This phenotype was generally opposite from that due to rab5 overexpression (Bucci et al., 1992 [this issue of *Cell*]), suggesting that the two

rab proteins help maintain a balance in membrane traffic between early endosomes and the plasma membrane.

Results

Stable Expression of Human rab4 and Tfn-R in CHO Cells

To avoid complications associated with the use of transient expression systems, we generated CHO cell lines stably transfected with human wild-type or mutant rab4. In the first mutant, the cdc2 kinase site at Ser-196 was changed to a glutamine (rab4-$S^{196}Q$). This mutant was not phosphorylated in mitotic cells and thus remained endosome associated throughout the cell cycle (van der Sluijs et al., 1992). The second mutant had an asparagine to isoleucine change in the GTP-binding domain of rab4 (rab4-$N^{121}I$) (Dever et al., 1987) and was found unable to bind GTP in vitro (not shown). The analogous mutant of sec4 in yeast acts as a dominant inhibitor of the endogenous protein (Walworth et al., 1989). Cells were transfected using the plasmid vector pFRSV, and rab4 expression was amplified by selection in methotrexate (Horwich et al., 1985). The resulting cell lines overproduced human rab4 relative to the endogenous protein by ~80-fold (wild type and $S^{196}Q$) and ~15-fold (rab4-$N^{121}I$ mutant). In each case, >90% of the rab4 produced remained membrane associated (van der Sluijs et al., 1992). Thus, even 80-fold overexpression did not lead to an accumulation of soluble rab4.

Localization of Transfected rab4 to Early Endosomes

To demonstrate that exogenous rab4 was localized to early endosomes, we cotransfected a cDNA encoding the human Tfn-R as an intrinsic marker of the early endocytic pathway. As shown in Figure 1, confocal microscopy demonstrated that wild-type rab4 (a), rab4-$S^{196}Q$ (d), or rab4-$N^{121}I$ (g) exhibited a punctate staining pattern that was similar to that detected by antibody to human Tfn-R (Figures 1b, 1e, and 1h). The extent of colocalization was tested by merging the confocal images in which coincident staining appeared yellow (Figures 1c, 1f, and 1i). Little if any red fluorescence was observed in the merged images, indicating that all of the detectable rab4-positive structures were also positive for Tfn-R. Some green fluorescence remained, however, indicating that not all Tfn-R-positive elements contained rab4. For example, staining corresponding to cell surface Tfn-R (e.g., the margins of cells shown in Figures 1b and 1h) remained green after the merge (Figures 1c and 1i). No colocalization was observed in cells stained for rab4 and lgp-B, an endogenous marker for late endosomes and lysosomes (Kornfeld and Mellman, 1989) (Figures 1i, 1j, and 1k), illustrating that little rab4 was found in late endocytic compartments even after overexpression.

Analysis of Endocytosis in Cells Overexpressing rab4

To define a possible role for rab4, we next analyzed the rab4 transfectants using three assays that measure different features of the endocytic pathway. In the first assay, we monitored the uptake and degradation of ^{35}S-labeled Semliki Forest virus (SFV), a well-characterized marker of endocytosis in CHO cells (Schmid et al., 1988, 1989). SFV binds to surface receptors, is internalized in clathrin-coated pits, and is transferred from early endosomes to late endosomes and lysosomes, where the virus is degraded. Since incoming virus fuses with the endosomal membrane, little if any SFV is recycled back to the plasma membrane. Thus, SFV provides a measure of internalization and vectorial transport to lysosomes.

In the second assay, we measured the uptake and accumulation of horseradish peroxidase (HRP), a marker of fluid phase endocytosis. Fluid phase markers are delivered to early endosomes and then either sequestered intracellularly by sorting to late endosomes or released back into the medium as a consequence of being contained within recycling vesicles formed mostly from early endosomes (Besterman et al., 1981; Griffiths et al., 1989; Steinman et al., 1983). Thus, unlike SFV, the accumulation of HRP reflects not only endocytosis but the sorting and recycling efficiency of early endosomes.

Finally, to investigate sorting and recycling in greater detail, we also monitored the uptake and recycling of receptor-bound Tfn. After internalization, Tfn is delivered to early endosomes where the acidic pH causes dissociation of Tfn-bound iron (Ciechanover et al., 1983; Dautry-Varsat et al., 1983; Hopkins and Trowbridge, 1983; Klausner et al., 1983). The complex is then thought to enter a population of less acidic recycling vesicles or tubules, which often accumulate in the Golgi region (McGraw et al., 1987; Stoorvogel et al., 1991; Yamashiro et al., 1984). The receptor is then returned to the plasma membrane, where iron-depleted Tfn is released into the medium. Thus, by monitoring the Tfn cycle, effects of rab4 expression on the constitutive pathway of receptor recycling can be determined.

Endocytosis and Degradation of SFV Is Not Affected by rab4 Expression

We first determined whether overexpression of rab4 affected receptor-mediated endocytosis of ^{35}S-SFV or its vectorial transport to late endosomes and lysosomes. As shown in Figure 2, overexpression of either wild-type or mutant rab4 (both rab4-$S^{196}Q$ and rab4-$N^{121}I$) had little effect on the rates of endocytosis or degradation of ^{35}S-SFV relative to nontransfected control cells. In each case, internalization was rapid ($t_{½}$ = <5 min), and the appearance of trichloroacetic acid–soluble ^{35}S, previously found to correlate with delivery to lysosomes and late endosomes (Schmid et al., 1988), could be detected after a lag of ~10 min. Thus, neither the initial rate of SFV internalization nor its subsequent transit to late endosomes and lysosomes was significantly affected by overexpression of wild-type or mutant rab4, suggesting that rab4 does not directly affect any of these steps.

Accumulation of Fluid Phase Markers Is Inhibited by Overexpression of Wild-Type rab4

We next examined the accumulation of HRP in the rab4 transfectants. As illustrated in Figure 3, nontransfected control cells and cells expressing either of the rab4 mu-

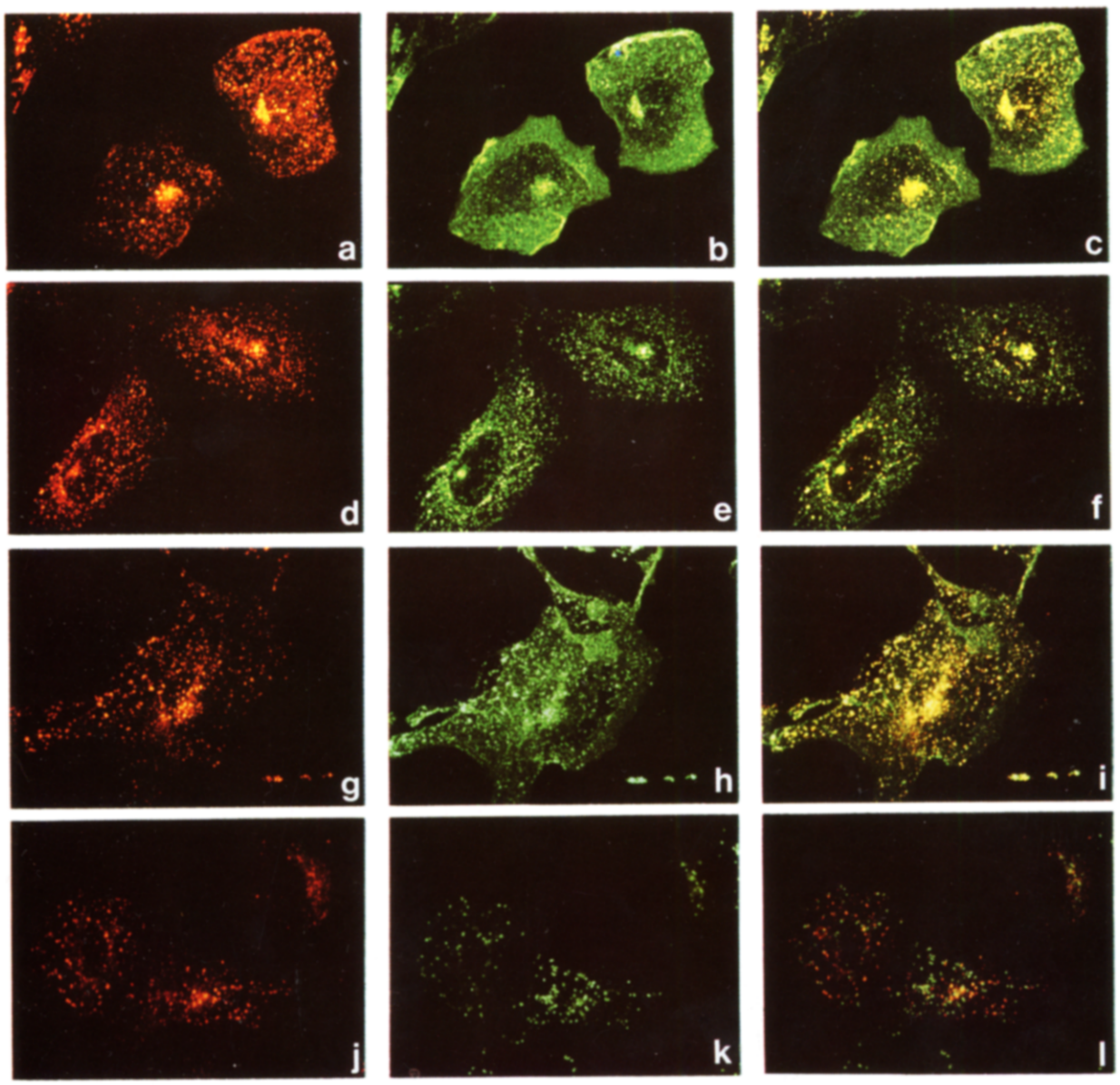


Figure 1. Transfected rab4 Colocalizes with the Human Tfn-R

Cells plated on glass coverslips were fixed with paraformaldehyde–lysine–periodate, permeabilized with saponin, incubated with an affinity-purified rabbit anti-rab4 antiserum ([a], [d], [g], and [j]) and a monoclonal antibody against the human Tfn-R ([b], [e], and [h]), and stained with tetramethylrhodamine B isothiocyanate–conjugated donkey anti-rabbit immunoglobulin G or FITC-conjugated goat anti-mouse antibody. Lysosomes and late endosomes were visualized with a monoclonal antibody against the hamster lysosomal membrane glycoprotein lgp95 (lgp-B) and a tetramethylrhodamine B isothiocyanate–conjugated goat anti-mouse antibody (k). To visualize the degree of colocalization of rab4 staining with either Tfn-R or lgp95 staining, the two images were merged by pairs ([c], [f], [i], and [l]). Colocalization was indicated when the red or green staining became more yellow.

tants exhibited comparable rates of HRP accumulation for at least 1 hr. After 2 hr, HRP uptake by cells expressing rab4-$S^{196}Q$ was, if anything, slightly increased relative to controls. In contrast, HRP accumulation in CHO cells overexpressing wild-type rab4 was substantially reduced by 2- to 3-fold at all time points. This reduction was likely to reflect a decreased ability of the cells to retain internalized HRP, as opposed to a decrease in the rate of endocytosis per se, because the initial rate of SFV internalization was unchanged by rab4 expression (see Figure 2); SFV and HRP have previously been shown to be internalized via the same endocytic coated vesicles in fibroblasts (Marsh and Helenius, 1980). Similar results were obtained with multiple independent rab4 transfectants. Moreover, the fact that the rab4-$S^{196}Q$ or rab4-$N^{121}I$ mutants were without effect indicated that the alteration in HRP uptake due to wild-type rab4 was not a nonspecific effect of transfection or of overexpression of early endosome-associated rab proteins.

Overexpression of rab4 Alters the Tfn-R Cycle

To analyze more directly the effect of rab4 overexpression on early endosome function, we next determined whether any aspect of Tfn-R recycling was altered by rab4 expres-

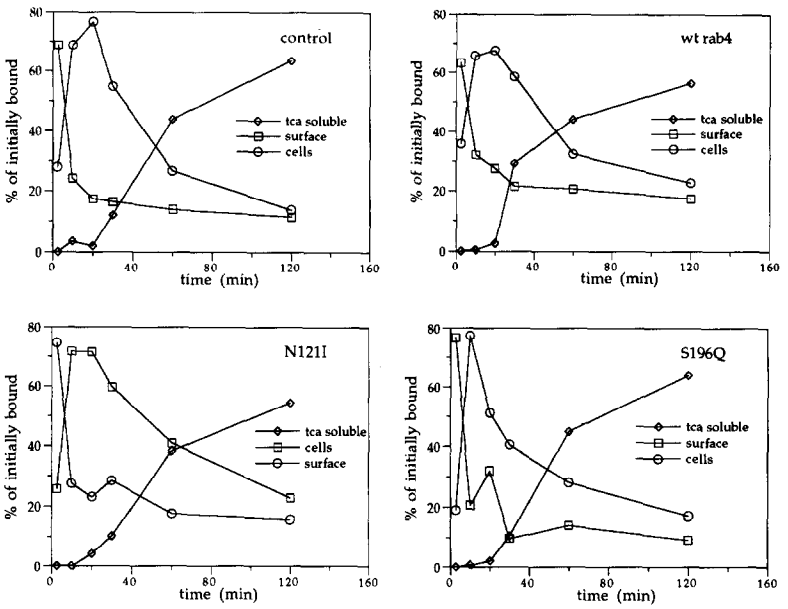


Figure 2. Internalization and Degradation of ^{35}S-Labeled SFV

Metabolically labeled SFV was prebound to triplicate cultures of non-rab4 transfected control cells or cells transfected with wild-type rab4, rab4-N^{121}I, or rab4-S^{196}Q for 2 hr at 4°C (all cells were transfected with human Tfn-R cDNA). After removing unbound virus, endocytosis was initiated by transferring the cells to 37°C for the indicated periods of time. Degradation was measured as trichloroacetic acid–soluble radioactivity released into the medium. Surface-bound (surface) and internalized (cells) ^{35}S-SFV were determined as amounts of radioactivity sensitive or resistant to removal from the cells following subtilisin treatment at 0°C. A minimum of 50,000 cpm was initially bound to each plate, and triplicates varied by 10%.

sion. We first measured the steady-state distribution of human Tfn-R on the plasma membrane. Cells were incubated in ^{125}I-human Tfn at 0°C or 37°C for 1.5 hr to determine surface versus total cell (surface plus intracellular) Tfn-R, respectively. Control experiments indicated that both binding and uptake reached steady-state levels within 60 min. The total number of Tfn-R labeled at 37°C varied by only 2- to 3-fold among the various cell lines, and endogenous CHO Tfn-R did not contribute (<10%) to the binding or uptake of the human ^{125}I-Tfn.

In cells not transfected with *rab4* cDNA, almost 80% of the total Tfn-R was found intracellularly, while only 20%–25% was present on the plasma membrane (Figure 4). In cells overexpressing wild-type rab4, however, this situation was reversed: 75%–80% of the total cell Tfn-R was now found on the cell surface, as opposed to only 20% found intracellularly (Figure 4). The distribution of Tfn-R in cells overexpressing the rab4-S^{196}Q mutant was indistinguishable from the nontransfected controls, while cells expressing the GTP binding–deficient rab4-N^{121}I mutant exhibited a partial alteration (~50% of the receptors found on the plasma membrane) (Figure 4). Thus, overexpression of wild-type rab4 and, to a lesser extent, the rab4-N^{121}I mutant greatly decreased the proportion of Tfn-R that was found in the intracellular pool.

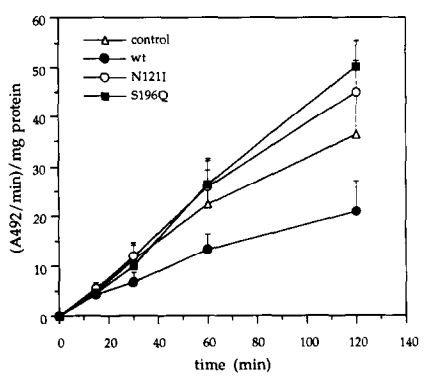


Figure 3. Endocytosis of HRP

Nontransfected control cells and cells transfected with wild-type or mutant rab4 were incubated in medium containing 2.5 mg/ml HRP at 37°C. At the indicated times, the cells were washed extensively on ice and lysed, and HRP was determined colorimetrically. Results are normalized to cell protein and represent the mean and standard deviation of three to four experiments.

Given the alteration in steady-state distribution of Tfn-R, we next determined the kinetics of Tfn uptake and release during a single round of the Tfn cycle. ^{125}I-Tfn was bound to cells at 0°C, unbound ligand was removed, and the cells were rapidly warmed in Tfn-free medium to 37°C. At various times, amounts of surface and intracellular ^{125}I-Tfn were determined; the fraction of ^{125}I-Tfn released into the medium was also monitored. As shown in Figure 6, in control cells transfected only with the human Tfn-R, ^{125}I-Tfn was rapidly cleared from the plasma membrane ($t_{1/2}$ = <5 min), accumulated transiently intracellularly (reaching a peak at ~5 min), and then released (presumably as iron-depleted apo-Tfn) into the medium as a consequence of Tfn-R recycling. Similar kinetics were observed after transfection of the rab4-S^{196}Q mutant.

In contrast, the kinetics of the Tfn cycle were significantly different in cells cotransfected with wild-type rab4 (Figure 5). The initial rate of ^{125}I-Tfn internalization was comparable with control cells (even at time points <2.5 min; not shown). However, the amounts of both surface-bound and intracellular ^{125}I-Tfn reached a plateau after 5 min with ~50% of the ligand on the surface and ~40% of the ligand intracellular. While these amounts decreased slowly with time as the fraction of ^{125}I-Tfn released into the medium slowly increased, after 30 min 80% of the ^{125}I-Tfn remained with the cells. This is in contrast with the <20% remaining with control cells after 30 min. It is unlikely that endocytosis of surface-bound ^{125}I-Tfn simply stopped after

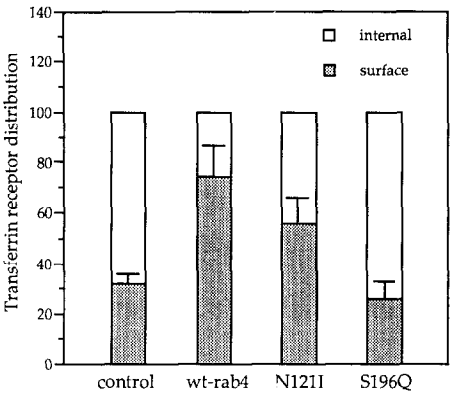


Figure 4. Distribution of Human Tfn-R's in rab4-Expressing Cells

After depleting endogenous Tfn by incubation in serum-free medium, CHO cells stably transfected with human Tfn-R, cotransfected with wild-type or mutant human rab4, or not transfected with rab4 (control) were incubated in medium containing ^{125}I-Tfn (2 μg/ml) for 60 min at either 0°C or 37°C. The plates were then transferred to ice, the cells lysed in Triton X-100, and cell-associated radioactivity determined. The percentage of surface or intracellular receptor-bound ^{125}I-Tfn was determined from the ratio of radioactivity bound at 0°C (surface) versus that bound and internalized at 37°C (total). All determinations were performed in triplicate and varied by <10%. Nonspecific binding and/or uptake (<5%) was determined by including 100-fold excess unlabeled Tfn during the incubation. The total amount of specific binding of ^{125}I-Tfn was between 0.44 × 10^5 and 1.31 × 10^5 cpm/well (the number of Tfn-R per well varied by 2- to 3-fold among the various cell lines used).

15 min or that only a fraction of surface receptors were capable of rapid internalization. Instead, these results suggested that surface and intracellular ^{125}I-Tfn reached a steady state, reflecting the continuous recycling of ^{125}I-Tfn-Tfn-R complexes in cells with a decreased intracellular receptor pool.

A similar but more complex phenotype was observed in cells expressing the rab4-N^{121}I mutant (Figure 5). In addition to a partial slowing of Tfn release, the initial rate of ^{125}I-Tfn internalization was also found to be ~2-fold slower than control cells. Thus, the rab4-N^{121}I mutant exerted a less selective effect on the Tfn cycle than wild-type rab4, suggesting that expression of a mutation that interferes with, as opposed to enhances, the function of endogenous rab4 exerts a more generalized disruption of the Tfn pathway. It is likely that the rab4 mutant alters the Tfn pathway by a mechanism distinct from that due to expression of the wild-type rab4.

rab4 Overexpression Prevents Tfn from Reaching Acidic Early Endosomes

The ability of wild-type rab4 overexpression to inhibit the release of ^{125}I-Tfn from transfected cells might reflect either an inhibition of Tfn-R recycling or an inhibition of the release of ^{125}I-Tfn from otherwise normally recycling receptors. Dissociation might be blocked if Tfn was returned to the plasma membrane without having discharged its iron intracellularly, since iron-bound Tfn dissociates less readily from Tfn-R (Dautry-Varsat et al., 1983; Klausner et al., 1983).

We first eliminated a possible defect in recycling per se by demonstrating that previously internalized Tfn-Tfn-R complexes were returned to the cell surface at equal rates in all cell lines. This was accomplished by allowing cells to internalize ^{125}I-Tfn derivatized with biotin via a cleavable disulfide linkage (biot-SS-^{125}I-Tfn) for 1 hr at 37°C, washing, and treating at 0°C with the impermeable reducing agent MESNA to selectively strip biotin from surface-bound but not intracellular biot-SS-^{125}I-Tfn. The cells were then incubated in prewarmed media containing excess unlabeled Tfn to facilitate dissociation even of recycled iron-bound Tfn (Klausner et al., 1984). After different periods of time, the cells were again rinsed with MESNA on ice and lysed, and intracellular biot-SS-^{125}I-Tfn was detected by adsorption to streptavidin beads. Using this approach, we found that previously internalized Tfn was re-

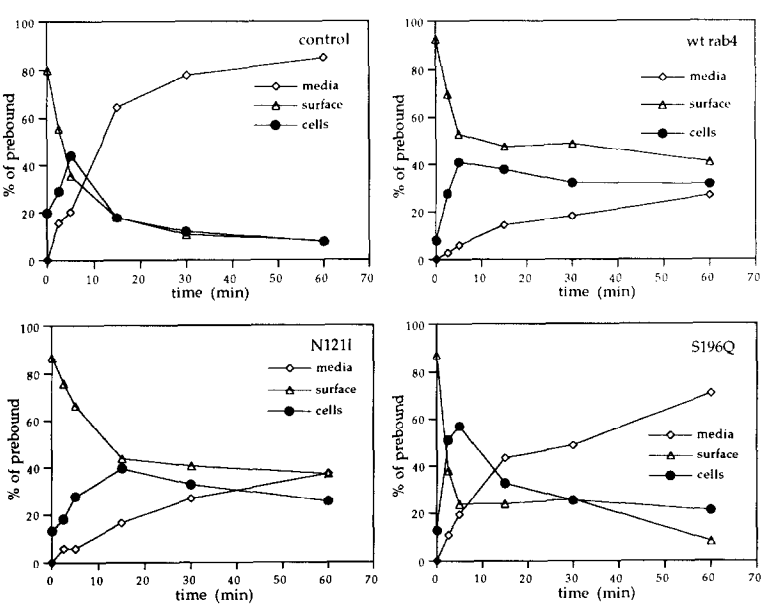


Figure 5. Receptor-Mediated Endocytosis and Recycling of ^{125}I-Tfn by rab4 Transfectants

After depleting endogenous Tfn, nontransfected control cells and rab4 transfectants were allowed to bind ^{125}I-Tfn on ice for 1 hr. After removal of unbound ligand, the cells were allowed to internalize the prebound ^{125}I-Tfn following rapid warming to 37°C. At the indicated time points, the medium was harvested to determine ^{125}I-Tfn release and the cells were returned to ice. Remaining surface-bound and intracellular ^{125}I-Tfn were measured by determining the accessibility of cell-associated radioactivity to elution by acid wash on ice. Nonspecific binding, uptake, and release were measured in the presence of 100-fold excess unlabeled Tfn and were <5%. All determinations were performed in triplicate and varied by <10%. At time 0, the specific binding of ^{125}I-Tfn was 0.6 × 10^5 to 3.3 × 10^5 cpm/well.

leased from all cell lines with similar kinetics (not shown), despite the fact that they exhibited significant differences in the absolute amounts of internalized Tfn.

Since recycled ^{125}I-Tfn was slow to be released from the transfectants in the absence of excess unlabeled Tfn, it may have returned to the cell surface still in its diferric iron-bound form. Accordingly, we reasoned that incoming ^{125}I-Tfn might not reach endosomes of sufficiently low pH to facilitate the dissociation of Tfn-bound iron. Accordingly, we next investigated the acidification properties of the endocytic compartments containing internalized Tfn. Endosome-enriched (plasma membrane–depleted) fractions were prepared from cells allowed to internalize fluorescein isothiocyanate (FITC)-Tfn for 1 hr at 37°C (Fuchs et al., 1989). The capacity of these vesicles for ATP-driven H^+ transport was then determined in vitro by monitoring by spectrofluorometry the pH-dependent decrease in FITC fluorescence. As expected, endosomes from control cells or from cells expressing the rab4-S^{196}Q mutant exhibited significant acidification upon addition of ATP (Figure 6A). These pH gradients were immediately reversed upon addition of the carboxylic ionophore nigericin, demonstrating that acidification occurred intravesicularly. In contrast, FITC-Tfn-containing endosomes from cells overexpressing wild-type rab4 or the rab4-N^{121}I mutant exhibited a marked reduction in both the rate and extent of acidification activity (Figure 6B). The small amount of ATP-driven H^+ transport observed was reversed by nigericin.

The acidification "defect" was specific for early Tfn-containing compartments. When cells were allowed to take up FITC-dextran for 1 hr to label late endosomes and lysosomes (Schmid et al., 1988), all cell lines exhibited equivalent amounts of in vitro acidification activity (Figure 6B). Thus, it is apparent that overexpression of wild-type rab4 (and the rab4-N^{121}I mutant) reduced the ability of Tfn to reach or accumulate in acidic early endosomes without causing a generalized defect in acidification. It was less likely that rab4 expression inhibited acidification directly because CHO cells with genetic defects in early endosome acidification do not exhibit the alterations in the Tfn cycle observed in the transfectants (Fuchs et al., 1989; Klausner et al., 1984; Roff et al., 1986; Schmid et al., 1989).

Consistent with the in vitro acidification results, cells expressing either the wild-type rab4 or the rab4-N^{121}I mutant accumulated iron at greatly reduced rates relative to control cells that did not exhibit defects in vitro (Figure 7). Thus, both in intact cells and in vitro, it was apparent that overexpression of wild-type rab4 reduced accumulation of Tfn in acidic endosomes. These results also emphasized that acidic pH in early, and not late, endosomes is required for iron discharge from Tfn.

rab4 Overexpression Alters the Morphology of Tfn-Containing Compartments

Since rab4 overexpression decreased the accumulation of Tfn in acidic endosomes, we next asked whether there were any alterations in the morphology of the endosomes in the transfectants. Cells were allowed to internalize HRP-Tfn for 1 hr at 37°C to label all compartments reached by Tfn (Stoorvogel et al., 1991). After fixation, Tfn

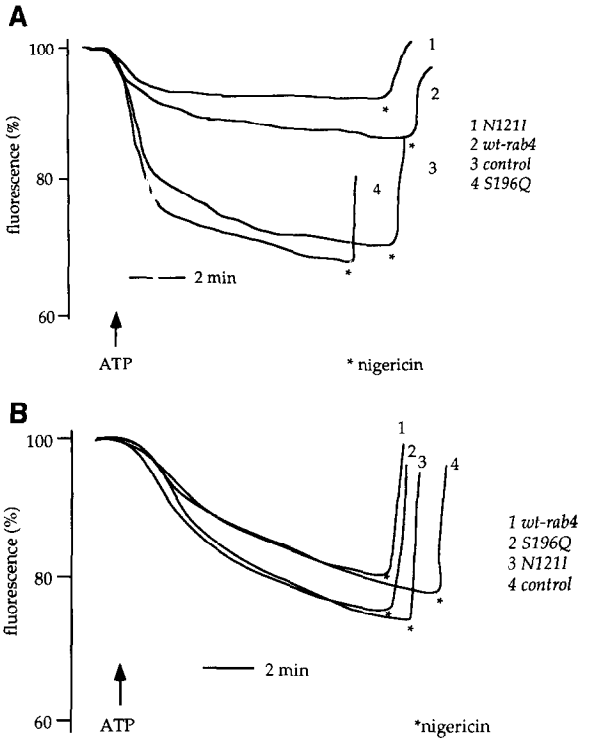


Figure 6. Acidification of Endosomes Isolated from rab4-Transfected Cells

After depletion of endogenous Tfn, the cells were incubated for 60 min with 20 μg/ml FITC-Tfn (A). Alternatively, 10 mg/ml FITC-dextran was internalized for 60 min (B). Cells were washed and endosome-enriched fractions prepared by centrifugation in sucrose density gradients. Following incubation in KCl-containing buffer on ice until ionic equilibrium was reached, endosomes were assayed for acidification activity after the addition of 0.5 mM ATP by monitoring the decrease in FITC fluorescence in a spectrofluorometer (Fuchs et al., 1989). All pH gradients were dissipated by the addition of nigericin (2.5 μM).

was visualized using diaminobenzidine–H_2O_2 and examined by electron microscopy. In control cells, HRP-Tfn was found in a population of small (<0.2 μm diameter) vesicles and tubules scattered throughout the cytoplasm and occasionally associated with the Golgi complex (Figure 8A). This localization was consistent with an accumulation of Tfn predominantly in endosomes and recycling vesicles as described previously (Hopkins et al., 1990; Tooze and Hollinshead, 1991; Yamashiro et al., 1984). A significantly different distribution was observed in cells overexpressing wild-type rab4. In addition to being found in small vesicles, HRP-Tfn was commonly observed in a distinct population of tubules and tubule clusters (Figure 8B). Interestingly, these tubules, as well as labeled vesicles, were often found in the vicinity of the Golgi complex (Figure 8B, inset). HRP-Tfn in cells expressing the rab4-N^{121}I mutant exhibited a phenotype similar to that found in the wild-type rab4 transfectants. However, in the rab4-N^{121}I transfected cells, the labeled structures seemed to be more evenly scattered throughout the cytoplasm, as opposed to being Golgi associated (Figure 8C). Moreover, some of the tubular profiles were elongated and studded with budding or fusing vesicles with the diameter (0.1–0.2 μm) of coated

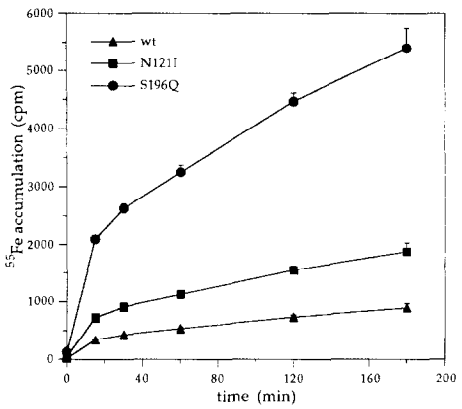


Figure 7. Kinetics of ^{55}Fe Accumulation from Internalized ^{55}Fe-Tfn

Endogenous Tfn was depleted as above, and transfectants were incubated in quadruplicate with 2.5 μg/ml ^{55}Fe-Tfn for the indicated times. The cells were washed with ice-cold PBS and solubilized in 1% Triton X-100 in PBS. ^{55}Fe in the samples was determined by liquid scintillation counting, and results were normalized to protein content and receptor expression.

vesicles (Figure 8C, inset). In both transfectants, a number of labeled vesicles of larger diameter (>0.5 μm) were also observed.

Preliminary quantitative estimates have confirmed that the Tfn–HRP-positive structures observed in either transfectant were morphologically different from those seen in control cells. They also appear reduced in overall surface area. While the identity or origin of these structures is not certain, it seems likely that accumulation of Tfn in these vesicles and tubules, as opposed to the more typical early endosomes, reflected the accumulation of FITC-Tfn in a compartment that was unable to exhibit efficient acidification.

Discussion

rab proteins are thought to play critical roles in regulating transport in the biosynthetic and endocytic pathways in mammalian cells. However, there is little direct evidence establishing the functional significance of more than one or two members of this increasingly large family. While important information has been obtained about rab protein function from cell-free assays of membrane transport (Gorvel et al., 1991; Plutner et al., 1990, 1991), this strategy is limited by the fact that, particularly in the endocytic pathway, it has not yet been possible to identify, much less reconstitute, all of the potential steps in which any one rab protein might be involved. In this paper, we have developed an alternative approach to analyze the function of the early endosome–associated protein rab4. By overexpressing wild-type or mutant rab4, we have found that this protein plays a critical role in the sorting functions of endosomes. While we have not identified the precise step controlled by rab4, we have directly demonstrated in intact cells that an endosome-associated rab protein does play an important role in the sorting or transit of Tfn-R and fluid phase markers through early endosomes. Moreover, the effect of rab4 overexpression appears to be specific for the recycling pathway, since the transport of SFV to late endocytic compartments was not altered.

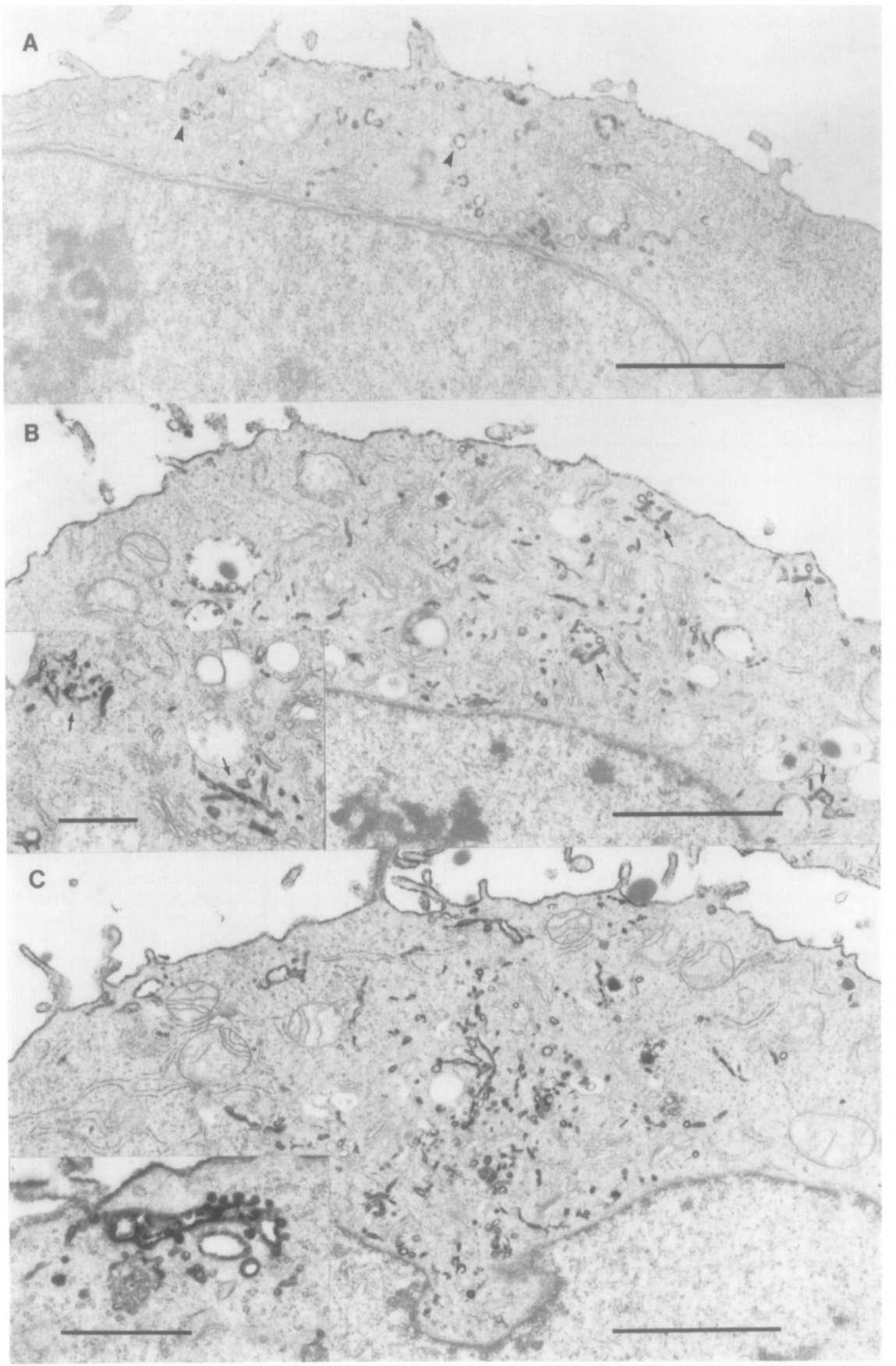
rab4 Expression Regulates the Pathway of Tfn-R Recycling

Since CHO cells contain their own endogenous rab4 (van der Sluijs et al., 1991), we relied on the ability to overexpress wild-type or mutant human rab4 and monitor alterations in the endocytic pathway. If endogenous rab4 were rate limiting for some endosome-related function, the overexpression of the wild-type protein might enhance the step(s) normally controlled by rab4. Conversely, a mutant deficient in GTP binding might interfere with the function of the endogenous protein, as did the analogous sec4 mutation on secretion in yeast cells (Walworth et al., 1989).

The effects of overexpression of wild-type rab4 were both selective and dramatic. The most striking alteration was the change in the steady-state distribution of Tfn-R between the plasma membrane and intracellular compartments. In nontransfected cells or cells transfected with the apparently inactive rab4-S^{196}Q mutant, up to 80% of the total cell Tfn-R was found intracellularly. Following expression of wild-type rab4, most of these receptors were found on the cell surface. This alteration was accomplished without changing either the initial rate of endocytosis or the recycling kinetics of preinternalized Tfn. Thus, an increase in the amount of rab4 must have affected a step that is normally rate limiting for the transit or processing of Tfn through intracellular compartments. Conceivably, such an alteration might reflect a decrease in the size of the endosomal compartment through which Tfn passes.

The normal pathway of Tfn-R recycling has been extensively studied and shown to involve the delivery of iron-bound Tfn to early endosomes where the acidic internal pH facilitates the discharge of one or both of the bound iron atoms. Interestingly, Tfn is then thought to exit early endosomes and enter a presumptive recycling compartment consisting of small vesicles and tubules with a relatively neutral internal pH (Stoorvogel et al., 1991; Yamashiro et al., 1984). Given the difference in pH, it seems likely that these vesicles comprise a compartment at least functionally distinct from acidic early endosomes. It is not yet clear whether the large intracellular pool of Tfn-R's normally results from accumulation of receptors in the presumptive recycling elements, in early endosomes, or in both. It is likely that the kinetics of entry into or through the recycling elements represents an important feature of the Tfn-R pathway since many other recycling receptors (e.g., the low density lipoprotein receptor) do not exhibit a comparably large internal receptor pool yet probably utilize the same pathway (Stoorvogel et al., 1991). Clearly, it will be of interest to determine the effect of rab4 on other rapidly recycling receptors to determine whether rab4 affects all receptors or Tfn-R selectively.

In any event, it is clear that overexpression of wild-type rab4 changes the pathway or site of accumulation of internalized Tfn relative to nontransfected control cells. By electron microscopy it was apparent that a greatly increased fraction of Tfn was found in small tubular clusters


A
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that, both in vitro and in intact cells, exhibited a marked reduction in their capacity for acidification. Relatively little Tfn was localized to the small diameter vesicles that were abundant in control cells. On the basis of morphology and the capacity to exhibit ATP-dependent H^+ transport in vitro, the structures labeled in control cells must correspond to early endosomes and recycling vesicles (Hopkins et al., 1990; Schmid et al., 1988, 1989; Tooze and Hollinshead, 1991).

While the identity of the small tubular clusters observed in the rab4 transfectants is not certain, it is conceivable that they represent elements of the nonacidic recycling compartment or the endosome-derived elements that give rise to that compartment. The effect of excess wild-type rab4, therefore, may be to alter the equilibrium distribution of Tfn-R between early endosomes and recycling vesicles by increasing the rate of removal of Tfn-R from acidic early endosomes (where Tfn no longer accumulates) or decreasing the effective size of the early endosome compartment. rab4 overexpression might also enhance the probability that incoming endocytic vesicles will be delivered directly to the recycling elements, thus avoiding fusion with acidic early endosomes in the first place. These events might result if rab4 controlled a rate-limiting event leading to the formation of or fusion with the recycling vesicles. Alternatively, wild-type rab4 might also act to oppose the fusion of incoming endocytic vesicles with early endosomes. Characterization of these and other possibilities will require much additional work both in intact cells and in vitro.

While we cannot completely eliminate the possibility that the observed alterations in endocytosis are secondary to an effect of rab4 expression on acidification, this seems unlikely for three reasons. First, CHO cell mutants with genetic defects in early endosome acidification do not exhibit any of the alterations in Tfn-R distribution or fluid phase endocytosis observed here (Fuchs et al., 1989; Klausner et al., 1984; Roff et al., 1986; Schmid et al., 1989). Second, treatment of cells with agents that disrupt endosomal pH, if anything, blocks recycling and increases the fraction of Tfn-R found intracellularly (Stein et al., 1984). Third, the fact that late endosomes continued to display normal ATP-dependent acidification demonstrated that these cells did not exhibit a generalized inactivation of H^+ transport.

The possibility that rab4 enhances the accumulation of elements involved in recycling is consistent with the endocytosis phenotypes exhibited by the wild-type rab4 transfectants. For example, the failure of incoming Tfn to reach, or to reside for a sufficient length of time, in acidic early endosomes would be expected to result in the observed inhibition of iron discharge. Accordingly, internalized Tfn would remain as iron-saturated iron-bound Tfn throughout its cycle. Moreover, the fact that iron-bound Tfn dissociates only slowly from plasma membrane receptors in the absence of competing iron-bound Tfn (Dautry-Varsat et al., 1983; Klausner et al., 1983) would be consistent with the failure of internalized ^{125}I-Tfn to be released from the transfectants unless excess unlabeled Tfn was included in the medium.

Similarly, a decrease in HRP accumulation would be expected by increasing the rate of recycling vesicle formation, reducing the overall size of the early endosome compartment, or decreasing access of incoming coated vesicles to early endosomes. Any of these alterations would be likely to reduce the sorting efficiency of early endosomes, resulting in an increase in the fractional loss of internalized fluid phase markers due to sequestration in a recycling compartment incapable of mediating delivery to late endosomes. Finally, even the absence of any clear effect on SFV uptake or delivery to lysosomes might reflect the fact that, unlike HRP or Tfn, internalized SFV is not subject to a significant amount of recycling (Marsh et al., 1983; Marsh and Helenius, 1980; Schmid et al., 1988, 1989). Either by cross-linking its receptors and/or by fusing with the endosomal membrane, SFV is likely to direct its own transport to lysosomes and prevent its inclusion in the recycling pathway. Consequently, alterations in the recycling functions of early endosomes should not affect the transit of SFV.

Understanding the effects of the rab4-N^{121}I mutant on endocytosis is made somewhat more difficult by the fact that its expression appeared to be less selective in its effects. Moreover, this mutant was expressed at levels only 15-fold over the endogenous wild-type rab4 (wild-type rab4 was 80-fold overexpressed). Although the rab4-N^{121}I transfectants exhibited a partial redistribution of Tfn-R to the plasma membrane, it is likely that the underlying mechanism was different from the wild-type transfectants since the rab4-N^{121}I cells also showed a partial reduction in the rate of Tfn internalization. The fact that similar reductions were not observed in HRP or SFV uptake could mean that the effects of rab4-N^{121}I were specific to the Tfn-R pathway or that other secondary effects were involved. Intracellular Tfn also accumulated in structures that were morphologically distinct from those observed in control cells or wild-type rab4 transfectants. Nevertheless, the Tfn-containing structures also exhibited a reduced capacity for ATP-dependent acidification. These vesicles might represent early endosome-derived elements en route but be pre-

Figure 8. Identification of Endocytic Compartments Containing HRP–Tfn

CHO cells were allowed to internalize HRP–Tfn for 1 hr at 37°C prior to fixation in 2% glutaraldehyde and visualization using diaminobenzidine cytochemistry. In control cells not transfected with *rab4* cDNA (A), labeling was observed in small (<0.1 μm diameter) vesicles and tubules scattered throughout the cytoplasm and in the Golgi region. In cells overexpressing wild-type rab4 (B), in addition to small vesicles, the reaction product was typically localized in tubular profiles. Many of these profiles were distinct clusters of tubules, which were seen less often in control cells (inset). Although the labeled structures were sometimes found throughout in the peripheral cytoplasm, many were clustered around the Golgi. In cells expressing the N^{121}I-rab4 mutant (C), similar tubular profiles were also observed. On occasion, these structures were closely associated with budding or fusing vesicles (inset). A small number of larger labeled structures were also present in both transfectants. The variability in diaminobenzidine staining was not judged to be significant since it varied between different samples of the same cell line. Bar = 0.5 μm.

vented from entering the recycling compartment. Alternatively, they might be derived from uncoated endocytic vesicles that also have a neutral internal pH (Schmid et al., 1989). It will be of interest to determine whether the budding or fusion images found in association with Tfn–HRP-containing tubules in the rab4-$N^{121}I$ transfectants are derived from coated vesicles.

rab Proteins and the Control of Endosome Function

Using kinetic analysis to identify the precise step affected by rab4 overexpression may prove difficult because even small alterations in the rate of Tfn-R recycling can have major effects on receptor fate and distribution (Weissman et al., 1986). However, it will now be possible to isolate and characterize the Tfn-containing vesicles that accumulate in the rab4 transfectants and to analyze their functional relationship to other endosomal elements in intact cells and in vitro. Irrespective of the step affected in the rab4 transfectants, it is striking that a rab protein was found in intact cells to exert significant and selective alterations in the pathway to which it has been morphologically associated. Recent results from Bucci et al. (1992) show that expression of another early endosome rab protein, rab5, exerts completely different effects on the endocytic pathway in intact cells. Using a transient expression system, overproduction of wild-type rab5 did not decrease but slightly enhanced endocytosis of HRP and Tfn. Moreover, intracellular HRP was found to accumulate in endosomes of increased diameter, unlike the small vesicular structures found in the rab4 transfectants. Since rab5 has already been shown to be involved in homotypic endosome fusion in vitro, the intact cell results suggest that overexpression of wild-type rab5 favors delivery of incoming endocytic tracers to early endosomes. Expression of a GTP binding–deficient form of rab5, in contrast, reduced HRP accumulation and resulted in its localization to smaller, fragmented endosomal elements. Thus, whatever their exact functions, the two early endosome rab proteins (rab4 and rab5) clearly have distinct and possibly opposing activities, despite being found in the same compartment.

Phenotypes associated with the overexpression of rab proteins may act by altering the rate-limiting steps in the pathways they operate. An illustration of how this might work has recently been provided in yeast cells (d'Enfert et al., 1991). Overexpression of sec12, a membrane protein involved in endoplasmic reticulum to Golgi transport, inhibited the formation of transport vesicles from the endoplasmic reticulum by titrating out the small GTP-binding protein SAR1. The inhibition was reversed by increasing SAR1 expression. By analogy, rab4 may interact with an abundant endosomal equivalent of sec12. If the putative interaction is required for generating recycling tubules in early endosomes, increasing rab4 expression would alter the equilibrium distribution of Tfn-R between early endosomes, recycling vesicles, and the plasma membrane.

Experimental Procedures

Construction of Mutant rab4

A cDNA encoding full-length rab4 was EcoRI restricted and cloned in the EcoRI site of Bluescript KS(+). Point mutations were generated by site-directed mutagenesis (Kunkel, 1985). rab4-$S^{196}Q$, in which Ser-196 was changed to a glutamine, is described elsewhere (van der Sluijs et al., 1992). rab4-$N^{121}I$, in which Asn-121 was changed to isoleucine, was generated using the mutagenic oligonucleotide primer 5′-GTC-CTT-CTT-GAT-TCC-ACA-AAG-3′. Mutations were confirmed by sequencing the entire coding region of both mutants using the dideoxy chain termination method (Sanger et al., 1977).

Stable Expression of *rab4* cDNAs in CHO Cells

cDNAs encoding wild-type rab4, $N^{121}I$, or $S^{196}Q$ were subcloned in the EcoRI site of pFRSV and transfected into CHO cells as previously described (Miettinen et al., 1989). Stably transfected cells were selected, cloned, and amplified in an α-minimal essential medium, supplemented with 10% fetal calf serum, 100 U/ml penicillin, 10 mg/ml streptomycin, and methotrexate (final concentration of 45 μM). Expression of protein was analyzed by immunofluorescence microscopy. Cells were subsequently transfected with a cDNA encoding the human Tfn-R in the expression vector pCB6 (Hunziker et al., 1991a), selected in medium containing 0.6 mg/ml G418 (GIBCO, Grand Island, New York), and screened by immunofluorescence microscopy for expression of both Tfn-R and rab4. Cells not expressing the human Tfn-R were removed by staining with the anti-Tfn-R antibody OKT9 using a FACS V fluorescence–activated cell sorter (Becton-Dickinson, Sunnyvale, California).

Fluid Phase Endocytosis Assay

Cells were grown to near confluency, washed twice with serum-free α-minimal essential medium, 10 mM HEPES, 0.1% bovine serum albumin (BSA) (internalization medium), and incubated for different periods of time with 2.5 mg/ml HRP at 37°C. The cells were washed three times with 5% BSA in cold internalization medium, three times with internalization medium, and three times with phosphate-buffered saline (PBS). Cells were then lysed with 1% Triton X-100 in PBS, and HRP was determined as described (van der Sluijs et al., 1991). Cell protein was determined using the Bio-Rad protein assay as per the manufacturer's instructions.

^{35}S-Labeled SFV Transport Assays

^{35}S-labeled SFV was produced as described (Marsh et al., 1983). ^{35}S-labeled SFV was bound at 8 pfu/cell for 2 hr on a rocking platform at 0°C in RPMI, 0.2% BSA, 20 mM HEPES (pH 6.8) (binding medium). Internalization (protease-resistant virus) and degradation (trichloroacetic acid–soluble radioactivity) were determined as described previously (Schmid et al., 1989). Each time point was performed in triplicate. Each of the cell lines bound equivalent amounts of virus per cell (corresponding to 50% of the added SFV).

Receptor-Mediated Endocytosis and Release of ^{125}I-Human Tfn

Human Tfn was iodinated as described (Podbilewicz and Mellman, 1990). For experiments, cells were plated in 12-well dishes 1–2 days prior to use, washed twice with PBS containing Ca^{2+} and Mg^{2+}, and incubated for 30 min at 37°C in Dulbecco's modified Eagle's medium, 0.2% BSA in a 37°C incubator to deplete endogenous Tfn. Binding was conducted by incubating monolayers (in triplicate) in ^{125}I-Tfn (2 μg/ml) in Dulbecco's modified Eagle's medium, 0.2% BSA, 20 mM HEPES (pH 7.4) (internalization medium) for 60 min on ice. Unbound Tfn was removed by seven washes with ice-cold PBS. Nonspecific binding was determined by including 100-fold excess of unlabeled Tfn and accounted for <5% of the total ^{125}I-Tfn bound. To measure internalization and recycling of the prebound ^{125}I-Tfn, the monolayers were transferred to a 37°C water bath for various periods of time. The plates were then returned to ice, the media harvested (to determine ^{125}I-Tfn release), and the monolayers washed in cold PBS (5 min) and then cold low pH buffer (150 mM NaCl, 10 mM acetic acid [pH 3.5]). This procedure was repeated three times and resulted in the removal of 98% of surface-bound ligand (from cells that had not been warmed to 37°C). The combined washes were used to determine the amount of surface-bound ^{125}I-Tfn. The cells were lysed in 1% Triton X-100 in PBS, and internalized ligand was defined as cell-associated ^{125}I counts per minute resistant to elution by the acid washes.

Cell-Free Acidification Assay

Cells were washed twice with serum-free α-minimal essential medium and depleted of endogenous Tfn by incubation at 37°C for 1 to 2 hr.

The cells were then washed once and incubated for 60 min at 37°C in internalization medium containing 20 μg/ml FITC-labeled Tfn (FITC-Tfn) or 10 mg/ml FITC-dextran (70,000 MW; Sigma) (Fuchs et al., 1989; Schmid et al., 1989). After washing on ice, the cells were homogenized, and membrane fractions enriched in endosomes were prepared by fractionation of the postnuclear supernatant on a discontinuous sucrose flotation gradient (Fuchs et al., 1989). This membrane fraction was greatly depleted in plasma membrane; <3% of the FITC-Tfn in this fraction was due to cell surface–bound ligand. Control experiments indicated that this amount of plasma membrane contamination did not alter the observed rate or extent of acidification. Membranes were equilibrated for 4.5 hr on ice in 20 mM HEPES–tetramethylammonium hydroxide (pH 7.4), 5 mM $MgSO_4$, and 150 mM KCl. Acidification was initiated by addition of ATP to 2.5 mM (from a 0.5 M stock, adjusted to pH 7.4 with KOH) and measured as quenching of fluorescein fluorescence in a Perkin-Elmer LS-5 spectrofluorometer (excitation wavelenth = 485 nm; emission wavelength = 515 nm). At the end of each experiment, reversibility of acidification was confirmed by dissipating pH gradients with 2.5 μM nigericin.

Assay for Intracellular Accumulation of ^{55}Fe

Human apo-Tfn (6 mg) was labeled with 1 mCi of $^{55}FeCl_3$ (New England Nuclear) essentially as described (Klausner et al., 1983). Double-transfected cells were plated in 12-well dishes 1–2 days prior to use, washed twice with PBS containing Ca^{2+} and Mg^{2+}, and depleted of endogenous Tfn. Uptake assays were performed in quadruplicate by incubating the cells with 2.5 μg/ml ^{55}Fe-Tfn at 37°C for various periods of time. The cells were washed seven times with ice-cold PBS and lysed in 1% Triton X-100 in PBS. Detergent lysates were mixed with Optifluor (Packard, Meriden, Connecticut), and intracellular ^{55}Fe was quantitated by liquid scintillation counting. Intracellular levels of ^{55}Fe were normalized with respect to total cell protein using the Bio-Rad protein assay, and the total amount of cell-associated ^{125}I-Tfn was measured after a 1 hr incubation with 2.5 μg/ml ^{125}I-Tfn.

Scanning Laser Confocal Immunofluorescence Microscopy

Transfects were plated on 12 mm glass coverslips and grown to 25%–50% confluency over 1–2 days. Immunofluorescence microscopy was exactly done as described (van der Sluijs et al., 1992) using affinity-purified rabbit anti-human rab4 antibody (Goud et al., 1990), the mouse monoclonal antibody OKT9 to Tfn-R (American Type Culture Collection, Bethesda, Maryland), or a mouse monoclonal anti-lgp-95 antibody (Harter and Mellman, 1992). The coverslips were viewed on a Bio-Rad MRC-600 confocal imaging system mounted on a Zeiss Axiovert microscope and directly photographed from a color monitor screen.

HRP Cytochemistry

Cells were depleted of endogenous Tfn, subsequently incubated with 5 μg/ml HRP–Tfn (Pierce Chemical Company, Rockford, Illinois) in Tfn internalization medium for 60 min at 37°C, and washed four times for 10 min with ice-cold internalization medium and once with ice-cold PBS. After fixation, the HRP reaction product was developed and examined on thin Epon sections as described (Hunziker et al., 1991b).

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